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Dated 26 July 2004

GB 0316089.2

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[ADP No. 08887986001]

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2.	Patent application number *(The Patent Office will fill in this part)*	0316089.2 — 0 9 JUL 2003
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	XO BIOSCIENCE LIMITED Lychgate House Main Street, Long Compton Warwickshire, CV36 5JJ, United Kingdom
	Patents ADP number *(if you know it)*	SECTION 30 (1977 ACT) APPLICATION FILED 7/6/04
	If the applicant is a corporate body, give the country/state of its incorporation	
4.	Title of the invention	DIFFERENTIATION METHOD
5.	Name of your agent *(if you have one)*	J.A. KEMP & CO.
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	14 South Square Gray's Inn London WC1R 5JJ
	Patents ADP number *(if you know it)*	26001

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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11. I/We request the grant of a patent on the basis of this application.

Signature J.A. Kemp & Co. Date 9 July 2003

J.A. KEMP & CO.

12. Name and daytime telephone number of person to contact in the United Kingdom

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DIFFERENTIATION METHOD

Field of the Invention

The present invention relates to the induction of differentiation of stem cells. The invention also relates to the reversal of differentiation of differentiated cells.

Background of the invention

Stem cells, and their application in regenerative medicine continue to dominate the scientific and lay press. Stem cells are undifferentiated cells that are capable of both self-renewal and also differentiation into one or more differentiated cell types. This dual ability to both divide to produce further stem cells and to differentiate means that stem cell populations can maintain their number whilst also giving rise to a large number of differentiated cells.

Stem cells reside in numerous locations of both plants and animals. Although much of the initial work carried out on stem cells focussed on embryonic stem cells, adult tissues also contain stem cells. Stems cells play essential roles such as in normal tissue repair. Stem cells also give rise to differentiated cells to replace those lost during the normal functioning of the body. For example, haematopoietic stem cells differentiate to give rise to various progenitor cells that in turn give rise to the various cells of the immune system. Thus as mature immune cells die they are replaced by new immune cells originating from the haematopoietic stem cells.

Stem cell populations can be routinely isolated for culture outside of the body or can be manipulated *in vivo*. Stem cells may be isolated from a variety of sources including normal adult tissues, pre-implantation embryos, foetal tissues (at various stages of development) and tumours. Both adult and embryonic stem cell lines have been established. Stem cell lines may be maintained in culture more-or-less indefinitely. Stem cell lines can also be manipulated in culture to introduce specific genetic modifications using techniques such as gene targeting.

Central to any application of stem cell technology is the ability to control differentiation of these cell types both *in vitro* and after transplantation to recipient humans, animals and plants. Although the ability of stem cells *in vivo* to differentiate is well known, the ability to artificially control the differentiation of

stem cells into mature cells is still in its infancy. Existing methods largely rely on exposing cultured stem cells to specific growth factors and/or growth conditions. Only a relatively limited number of specific differentiated cell types can be produced using such methods.

Summary of invention

The present invention is concerned with the control of differentiation. The present inventors have found that it is possible to induce stem cells to differentiate into a desired differentiated cell type and conversely that it is possible to reverse the differentiation of differentiated cells to provide stem cells. This is achieved by providing specific RNA sequences to the target cells.

The ability to produce differentiated cells from stem cells means that large numbers of desired differentiated cells can be obtained. In addition, the ability to produce stem cells from differentiated cells offers a much simpler, less labour intensive and less invasive way to provide stem cells in comparison to methods where stem cells have to be isolated directly. It also means that highly pluripotent stem cells may be obtained from adult tissues, making the use of embryonic tissues unnecessary. By combining the techniques for producing stem cells with those for differentiating them, large numbers of desired differentiated cells can be produced. The methods and medicaments of the invention mean that differentiation can be controlled both in tissue culture and in intact animals.

Accordingly, the present invention provides a method of inducing *in vitro* totipotent or pluripotent stem cells of a stem cell line or derived from a tissue of an animal or plant to differentiate into one or more desired cell types, which comprises providing isolated RNA comprising RNA extractable from tissue or cells comprising said desired cell type(s) to a cell culture of said stem cells under conditions whereby the desired differentiation of said stem cells is achieved. The stem cells employed may, in particular, be adult stem cells.

The invention also provides cells obtained by the above method. The differentiated cells may be used in the manufacture of medicaments for treating a number of disorders. Thus in a further aspect the invention provides for the use of the ~~cells in the manufacture of a medicament for use in improving or rectifying tissue or~~

cellular damage or a genetic disease. In some cases the isolated RNA itself may be used to induce differentiation *in situ*. Thus in another aspect the invention also provides for the use of the RNA capable of inducing differentiation of stem cells in the manufacture of a medicament for use in improving or rectifying tissue or cellular damage or a genetic disease.

In a further aspect the invention also provides a method for obtaining stem cells. Thus the invention provides a method of reversing *in vitro* the differentiation of differentiated cells of a cell line or obtained from the tissue of an animal or a plant to produce a desired type or types of totipotent or pluripotent stem cell(s) or stem cell line(s), which comprises providing isolated RNA comprising RNA extractable from the desired type(s) of stem cell or stem cell line to a cell culture of said differentiated cells whereby the desired reversal of differentiation of the differentiated cells into said type(s) of stem cell or stem cell line type(s) is achieved. The invention provides stem cells obtained using such methods. The invention also provides for the use of such cells in the manufacture of a medicament for use in improving or rectifying tissue or cellular damage or a genetic disease

The stem cells obtained using the methods of the invention may be induced to differentiate using a method in accordance with the invention. Thus in a further aspect the invention provides an *in vitro* method of producing differentiated cells, which comprises:

(a) performing a method in accordance with the invention to produce stem cells or a stem cell line from differentiated cells;

(b) performing a method in accordance with the invention on the stem cells or stem cell line to produce differentiated cells.

The invention also provides cells obtained by such methods. The cells obtained may be used to treat a number of diseases. Thus the invention also provides for the use of such cells in the manufacture of a medicament for use in improving or rectifying tissue or cellular damage or a genetic disease.

In some cases desired genetic modifications may be introduced into the stem cells.

Brief Description of the Figure

Figure 1: The effects of brain RNA differentiated stem cells on age related damage to the rat brain assessed by spatial learning and memory performance of recipient animals. Ex-breeder male rats aged between 468 to 506 days were given intravenously either untreated bone marrow stem cells or bone marrow stem cells treated with brain RNA extract. The results for control rats that received untreated stem cells (closed boxes) and those for experimental rats that received brain treated stem cells (open circles) are shown. The results show a remarkable increase in learning ability in the experimental rats.

Detailed description

The invention provides methods and medicaments for the controlled manipulation of any stem cell to induce the stem cell to differentiate into a desired differentiated cell type. In addition, the invention also provides methods for inducing the reversal of differentiation of a differentiated cell to provide a stem cell. The two methods may be combined so that a stem cell can be obtained from a differentiated cell and then differentiated to provide differentiated cells. Prior to the latter differentiation, the stem cells may be expanded in number and/or manipulated in a desired fashion for example to introduce a desired genetic modification.

A stem cell may, for example, be induced to differentiate in order to achieve a specific terminal differentiated state. Using the methods of the invention it is also possible to ensure that the differentiated cells are immunologically compatible with the intended recipient. The ability to chose what type of cell to induce the stem cell to differentiate into means that it is possible to produce a variety of different cell types from a single stem line or stem cell line. The RNA molecules of the invention or differentiated cells types obtained may be employed in the manufacture of medicament for treating various disorders. In particular they may be used in the manufacture of medicaments for use in improving or rectifying tissue or cellular damage or a genetic disease.

Stem cells

The invention may be used to produce or differentiate any suitable stem cell. A stem cell is generally understood to be a cell capable of self-renewal that is also capable of differentiation into one or more specific differentiated cell type(s). Stem cells may be pluripotent, that is they may be capable of giving rise to a plurality of different differentiated cell types. In some cases the stems cells may be totipotent, that is they may be capable of giving rise to all of the different cell types of the organism that they are derived from. The invention is applicable to pluripotent stem cells or totipotent stem cells.

In a particularly preferred embodiment the invention is used to differentiate or obtain adult stem cells. Stem cells are known to occur in a number of locations in the animal body. Stem differentiated or obtained cells may be those from any of the organs and tissues in which stem cells are present. Examples include stem cells from the bone marrow, haematopoietic system, neuronal system, the brain, muscle stem cells or umbilical cord stem cells. The stem cells may in particular be bone marrow stromal stem cells, neuronal stem cells or haematopoietic stem cells, in a preferred case they may be bone marrow stromal stem cells or neuronal stem cells. In particular when the methods of the invention are used to induce differentiation of a stem cell, the stem cell is a bone marrow stromal cell.

The stem cells may be plant or animal stem cells. In a preferred case, the stem cells will be animal stem cells and preferably mammalian stem cells. In one preferred embodiment, the stem cells may be human stem cells. Alternatively, the stem cells may be from a non-human animal and in particular from a non-human mammal. The stem cells may be those of a domestic animal or an agriculturally important animal. The animal may, for example, be a sheep, pig, cow, bull, or poultry bird or other commercially farmed animal. The animal may be a dog, cat, or bird and in particular from a domesticated animal. The animal may be a monkey such as a non-human primate. For example, the primate may be a chimpanzee, gorilla, or orangutan. The stem cells may be rodent stem cells. For example, the stem cells may be from a mouse, rat, or hamster stem cell.

In many cases the differentiated cells may be intended for the manufacture of medicaments to treat a subject. In such cases the stem cells may be from the intended

recipient. This may particularly be the case where the stems cells are obtained using the methods of the invention in order to reverse the differentiation of a differentiated cell to provide a stem cell. In other cases the stem cells may originate from a different subject, but be chosen to be immunologically compatible with the intended recipient. In some cases the stem cells may be from a relation of the intended recipient such as a sibling, half-sibling, parent or child, and in particular from a sibling. The stem cells may be from an unrelated subject who has been tissue typed and found to have a immunological profile which will result in no immune response or only a low immune response from the intended recipient which is not detrimental to the subject. However, in many cases the stem cells, or the differentiated cells used to generate the stem cells, may be from an unrelated subject as the invention may be used to render the stem cell immunologically compatible with the intended recipient.

In some cases the stem cells may be embryonic stem cells or foetal stem cells. The embryonic or foetal stem cells may be pluripotent stems cells and particularly totipotent stem cells. The cells may be from any stage of development, in particular they may be isolated from the inner cell mass of a blastocyst. The embryonic or foetal stem cells may be from, or derived from, any of the organisms mentioned herein. The embryonic or foetal stem cells may be human stem cells or non-human stem cells and in particular non-human animal stem cells. The embryonic or foetal stem cells may be rodent stem cells and may in particular be mouse embryonic stem cells. In some cases the embryonic or foetal stem cells may be recovered and then used in the manufacture of medicaments to treat the same subject, typically at some stage in their life. In a preferred embodiment, where embryonic or foetal stem cells are employed, they will be from already established foetal or embryonic stem cell lines. This will particularly be the case for human cells. In some cases the stem cells may be obtained from, or derived from, extra-embryonic tissues. The stem cells may be obtained from the umbilical cord and in particular from umbilical cord blood.

The invention is also applicable to stem cell lines. Stem cell lines are generally stem cell populations that have been isolated from an organism and maintained in culture. Thus the invention may be applied to stem cell lines including adult, foetal and embryonic stem cell lines. The stem cell lines may be a clonal stem cell line i.e. they may have originated from a single stem cell. In one preferred

embodiment the invention may be applied to existing stem cell lines, particularly to existing embryonic and foetal stem cell lines. In other cases the invention may be applied to a newly established stem cell line.

The stem cells may be an existing stem cell line. Examples of stem existing stem cell lines which may be used in the invention include the human embryonic stem cell line provided by Geron and the neural stem cell line provided by Reneuron. In a preferred case the stem cell line may be one which is a freely available stem cell access to which is open and in particular such an existing stem cell line.

In the case of human embryonic stem cell lines, in a preferred case a pre-existing stem cell line will be used. In a particularly preferred embodiment of the invention, where a human embryonic stem cell line is used, the cell line may be one where the derivation process (which begins with the destruction of the embryo) was initiated prior to 9:00 p.m. EDT on August 9, 2001. Preferably human embryonic stem cell lines may be ones created from embryos donated for reproductive purposes which were no longer needed for the original purpose, because, for example, they were surplus to requirements. Preferably informed consent will have been obtained for the use of the embryos to create the cell line. In a preferred case, the human embryonic stem cell line employed will meet the requirements announced by President Bush on 9 August 2001 as being necessary for obtaining US federal funding for embryonic stem cell research. These include the stem cell lines recognised as meeting the requirements from BresaGen Inc. of Australia; CyThera Inc.; the Karolinska Institute of Stockholm, Sweden ; Monash University of Melbourne, Australia; National Centre for Biological Sciences of Bangalore, India; Reliance Life Sciences of Mumbai, India; Technion-Israel Institute of Technology of Haifa, Israel; the University of California at San Francisco; Goteborg University of Goteborg, Sweden; and the Wisconsin Alumni Research Foundation.

Reference herein to stem cell generally includes the embodiment mentioned also being applicable to stem cell lines unless, for example, it is evident that the target cells are freshly isolated stem cells or the stem cells are resident stem cells *in vivo*. The invention is applicable to freshly isolated stem cells and also to cell populations comprising stem cells. The invention may also be used to control the differentiation of stem cells *in vivo*.

An initial step in the methods of the invention may be the isolation of suitable stem cells. Methods for isolating particular types of stem cells are well known in the art and may be used to obtain stem cells for use in the invention. The methods may, for example, be used to recover stem cells from the intended recipients of the medicaments of the invention. Cell surface markers characteristic of stem cells may be used to isolate the stem cells, for example, by cell sorting. Stem cells may be obtained from any of the types of subjects mentioned herein and in particular from those suffering from any of the disorders mentioned herein.

In some preferred embodiments stems cells may be obtained by using the methods of the invention to reverse the differentiation of differentiated cells to give stem cells. In particular, differentiated cells may be recovered from a subject, treated *in vitro* in order to produce stem cells, the stem cells obtained may then be manipulated as desired and differentiated before return to the subject. As stem cells typically represent a very small minority of the cells present in an individual such an approach may be preferable. It may also mean that stem cells are more easily derivable from specific individuals and may eliminate the need for embryonic stem cells. In addition, typically such an approach will be less labour intensive and expensive than methods for isolating the stem cells themselves. In some cases, the stem cells may be isolated from a subject, differentiated *in vitro* and then returned to the same subject. Such *ex vivo* methods are particularly preferred.

In some cases the target stem cells may be *in situ*, that is they may be present in a subject. Thus in a further aspect the invention provides for the use of an RNA in accordance with the invention which is capable of inducing differentiation of stem cells in the manufacture of a medicament for use in improving or rectifying tissue or cellular damage or a genetic disease. Such a method may be used for treating a degenerative brain disease or brain or spinal cord injury. It may also be used for the treatment of a disease selected from liver disease, heart disease, skeletal or cardiac muscle disease or type I diabetes. Furthermore, it may be used to counteract age-related degenerative disease.

In such embodiments the stem cells may be any of the types of stem cells mentioned herein and may be in any of the organisms mentioned herein. The target stem cells may be present in any of the organs, tissues or cell populations of the body

in which stem cells exist, including any of those mentioned herein. The target stem cells will typically be resident stem cells naturally occurring in the subject, but in some cases stem cells produced using the methods of the invention may be transferred into the subject and then induced to differentiate by transfer of RNA.

Various techniques for isolating, maintaining, expanding, characterising and manipulating stem cells in culture are known and may be employed. In some cases genetic modifications may be introduced into the genomes of the stem cells. Stem cells lend themselves to such manipulation as clonal lines can be established and readily screened using techniques such as PCR or Southern blotting. Techniques such as gene targeting or random integration may be used to introduce changes into the genome of the cells.

In some instances the stem cells may originate from an individual with a genetic defect. Modifications may then be made to correct or ameliorate the defect. For example, a functional copy of missing or defective gene may be introduced into the genome of the cell. Gene targeting may be used to introduce desired specific changes and in particular to modify a defective gene to render it normal. Site-specific recombinases may be used to remove selective markers involved in the gene targeting. In a particular preferred embodiment, differentiated cells will be obtained from an individual with a genetic defect, stem cells obtained from the differentiated cells using the methods of the invention, the genetic defect corrected or ameliorated and then either the stem cells or differentiated cells obtained from them will be used in the manufacture of medicaments for treating the original subject.

In some cases the stem cells may be chosen because they have a specific genotype. For example the stem cells may be intended to produce differentiated cells to treat a subject with a genetic defect. The stem cells may lack the genetic defect. For example, the stem cells may be obtained from, or produced from differentiated cells obtained from, a relation of the subject who lacks the defect. For example, the cells may be derived from a sibling who does not have the disorder. In a preferred case the methods of the invention may be used to render the cells immunocompatible or more immunocompatible with the intended host.

RNA molecules

In order to produce the desired changes in differentiation the invention employs specific RNA sequences. The RNA employed is one that comprises RNA extractable from tissues or cells comprising the cell type that it is desired to induce the target cell to become. Thus in the case where the aim is to induce differentiation of a stem cell into a desired differentiated cell type, the RNA provided to the target cell is typically an isolated RNA comprising RNA extractable from tissue or cells comprising the desired differentiated cell type or types. In cases where it is desired to reverse the differentiation of a differentiated cell to a desired stem cell type, the RNA provided is typically an isolated RNA comprising RNA extractable from the desired stem cell type or types which it is wished to obtained.

Typically, the RNA will comprise a heterogeneous population of species of different RNA molecules. In a preferred embodiment the RNA will comprise an RNA extract of tissues or cells comprising the desired cell type or types, in particular the RNA may comprise, or consist essentially of, an extract from the desired cell types. Thus preferably an RNA rich extract is prepared from donor material. The donor material may, for example, be an organotypic source obtained post mortem. For example the RNA extract may be from an organ or tissue or cells isolated from an organ or a tissue such as the brain, spine, heart, kidney, spleen , skin, the gastrointestinal tract or liver. The extract may be from a cell line of specific chosen phenotype, a primary cell culture, or a donor tissue of specific immunological profile.

Typically the RNA will comprise RNA that is extractable from the same species as the target cell to be treated. Thus in cases where the target cell to which the RNA will be provided is an animal cell, the RNA will usually comprise RNA extractable from an animal cell and in particular from the same species of animal. Similarly, where the target cell is a plant cell, usually the RNA will comprise an RNA extractable from a plant cell and typically a plant cell of the same species as the target cell. The RNA may comprise RNA extractable from any of the organisms or groups of organisms mentioned herein. The RNA may comprise RNA extractable from any of the stem cell types or differentiated cell types mentioned herein.

The induction of differentiation using the RNA in accordance with the invention as discussed above may result in the target cell also adopting an immunological profile similar or the same as that of the organism from which the RNA is extractable from. Thus the invention may be used to change the immunological profile of a target cell in a desired manner. This may be used to ensure that the cells produced, or products produced from them, have a specific immunological profile. In particular, the RNA provided to the target cells may therefore be chosen so that the resultant cells, or products from them, have an immunological profile so that they are not immunogenic in the intended recipient or produce a minor immune response which is not significant and that preferably does not result in a detrimental phenotype. Thus the RNA provided may in a preferred case be an RNA extractable from, and particularly an RNA extracted from, cells or tissues of the intended recipient or an immunologically compatible subject.

The ability to change the immunological profile of a cell may mean that the stem cells or differentiated cells to which the RNA is provided do not themselves have to necessarily be immunologically compatible with the intended recipient. This means that stem cells may not necessarily have to be isolated from the intended recipient and, for example, already existing stem cells or stem cells from a more convenient source may be used. It may also mean that stem cells with a specific desired genotype may be employed and converted to a compatible immunological profile. For example, the intended recipient may have a genetic defect, whereas the stem cells or differentiated cells to which the RNA is provided may be from a different subject that does not have the same defect. Using the invention the donor cells may be rendered immunologically compatible to the intended recipient and also compensate for the genetic defect.

Various techniques exist for the extraction of donor RNA. Such techniques may be used to obtain the RNA to be provided to the target cells. Alternatively such techniques may be used to provide RNA to identify the sequences of the necessary RNA molecules that may then be synthesised artificially. In some cases, the RNA may be an artificial or synthetic RNA or an RNA analogue based on the sequence of the extractable sequences. The analogue may be one chosen for its stability or ability to enter the target cell or other desirable properties.

RNA may, for example, be prepared from a donor source by either cold or hot phenol extraction methodologies. Alternatively, the RNA may be sourced from specific tissues or cells by employing commercially available kits and in particular those that are based on the denaturing of protein and separation of RNA via centrifugation. For example, in one preferred protocol, cold phenol extraction, primary donor tissue or cells is/are homogenised in a volume of physiological saline. An equal volume of 95% saturated phenol is added and initially centrifuged at 18,000rpm in an ultra centrifuge for 30 minutes. The aqueous phase is retained and brought to a concentration of 0.1M $MgCl_2$ solution by the addition of 1M $MgCl_2$. Two volumes of ethanol are then added and this is allowed to precipitate for approximately 30 minutes. A final spin at 6,000rpm for 15 minutes produces an RNA rich precipitate which is retained and stored under ethanol. Alternatively, active RNA rich extracts may be prepared with any of the commercially available RNA extraction kits (such as, for example, RNAzol™).

In some cases a specific fraction of an RNA extract may be employed. For example, the RNA population may be fractionated on the basis of size and a particular weight range of RNA species provided to the target cell. In some embodiments the RNA employed may comprise or consist of the mRNA sequences present in the extractable sequences. In some cases the RNA may comprise a mixture of sequences extractable from different cell types or tissues. For example, the RNA species may comprise a mixture of sequences extractable from two, three, four, five or more different cell types. In cases where it is desired to differentiate a stem cell, the RNA may, for example, be extractable from different cell types to produce a differentiated cell with characteristics of both cell types. In cases where the RNA is to be provided to a target cell that has a genetic defect, the RNA may be a mixture of sequences extractable from cells comprising and lacking the defect. For example, the RNA may comprise a blend of RNA extracts from cells from the subject with the defect and cells of the same type from another subject that lack the defect. In some cases specific sequences that are extractable from the desired cell type may not be present. For example, the transcript of a defective gene may be removed. The removal of specific sequences may, for example, be achieved, by selective

degradation or by hybridisation. Ribozymes may be used to cleave specific sequences.

Specific sequences may be added to or removed from the extractable sequences. For example, in some cases the RNA may originate from the subject intended to be the eventual recipient of the cells produced and the subject may lack a specific gene sequence or have a defective gene sequence. In such cases an additional RNA corresponding to an RNA encoding the expression product of the missing or defective gene may be added to the extract. In such cases, the defective RNA sequences may be removed or selectively degraded

In cases where the RNA is one extractable from a stem cell, preferred stem cells include any of those mentioned herein and in particular adult stem cells. The stem cell may, for example, be a haematopoietic, bone marrow stromal or neuronal stem cell. In cases where the RNA is one extractable from a differentiated cell, the differentiated cell may be any differentiated cell and may be in particular an adult differentiated cell. In a preferred embodiment the differentiated cell may be selected from a bone marrow cell, a neuronal cell, or a haematopoietic cell. The differentiated cell may be from any mammalian organ for example such as the kidney, liver, heart, central nervous system, reproductive organ or other organ.

The amount of RNA provided to the target cells will be sufficient to bring about the necessary desired differentiation. For example the concentration of RNA may be from 10ng to 5mg/ml, preferably from 100 ng/ml to 2.5 mg/ml, more preferably from 1μg/ml to 500 μg /ml, even more preferably from 5 μg/ml to 100 μg/ml and still more preferably from 10 to 50 μg/ml. In a particularly preferred case the RNA concentration may be from 15 to 40 μg/ml, preferably from 20 to 35 μg/ml and in particular may be 25 μg/ml. These concentrations may apply to *in vitro* or *in vivo* applications. In some cases, a total of 100 ng to 0.1 g, preferably from 1 μg to 50 mg, more preferably from 100 μg to 10 mg, still more preferably from 250 μg to 1 mg of RNA may be administered. Any suitable concentration and/or amount of RNA may be provided. A wide range of concentrations and/or amounts of RNA may be employed and the precise concentration and/or amount may be varied according to the method of delivery of the RNA to the target cells or tissues, the source of the RNA and whether the RNA is provided *in vitro* or *in vivo*

Provision of RNA to target cells

The RNA may be provided to the target cells *in vitro* or alternatively the RNA may be used in the manufacture of medicaments for the provision of the RNA to the target cells *in situ.* In the case of plants the invention provides methods for providing the RNA to the target cells both *in vitro* and *in vivo*. The RNA may be provided to the target cells by any suitable technique.

A number of methods for the provision of nucleic acid molecules to cells are known and these may be employed. For example, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection, transduction using viral particles and microinjection. The calcium phosphate precipitation method of Graham and van der Eb, Virology 52:456-457 (1978) may be employed. General aspects of mammalian cell host system transformations have been described in U.S. Patent No. 4,399,216 and may be employed. For various techniques for transforming mammalian cells, see Keown *et al.*, Methods in Enzymology, 185:527 537 (1990) and Mansour *et al.*, Nature 336:348-352 (1988). In some cases the RNA may simply be added to the culture medium of the cells for a suitable period of time. For example, the cells and RNA may be cultured together for from 1 minute to 10 days, preferably from 1 hour to 5 days, more preferably from 6 hours to 2 days. In a preferred embodiment the RNA may be cultured with the cells for 12 or 24 hours and in particular for 12 hours. Similar time periods may be employed where the RNA is provided in the form of liposomes comprising the RNA sequences.

In other embodiments the RNA may be used in the manufacture of medicaments which will allow *in vivo* provision of the RNA to stem cells. In such cases the RNA is typically formulated so that the medicament is in a suitable form for administration to the intended subject.

The medicament may be in a form where the RNA is in liposomes to facilitate delivery or alternatively encapsulated within viral particles. The RNA may be present as naked RNA molecules or RNA molecules complexed with proteins and in particular proteins known to increase uptake of nucleic acids into cells.

The medicament comprising the RNA may be delivered by any suitable route. For example, the medicament may be administered parenterally and may be delivered by an intravenous, intra-arterial, intramuscular, intraperitoneal, topical or other appropriate administration route. The medicament may be administered directly to the desired organ or tissue or may be administered systemically. In particular preferred routes of administration include via direct organ injection, vascular access, or via intra-muscular, intra-peritoneal, or sub-cutaneous routes. The RNA may be formulated in such a way as to facilitate delivery to the target cells.

The RNA may be provided on metallic particles. In some cases the medicament may be intended to administered so that naked RNA is provided to the target cells. In cases where the RNA is provided present in liposomes or other particles, there may be targeting molecules present on the surface of the particles to allow targeting to the intended stem cells. For example, the particles may comprise ligands for receptors on the target stem cells or target differentiated cells.

The various RNA preparations used to provide the RNA discussed herein to the target cell may also comprise agents to increase the stability of the RNA, For example, they may comprise RNAse inhibitors. The RNA preparations may also have been treated to remove other kinds molecules, for example protease or DNase treatment may have been used to remove protein and/or DNA.

In vitro methods for reversing the differentiation of differentiated cells in order to provide stem cells

The invention provides methods for reversing the differentiation of differentiated cells to produce stem cells. The invention thus provides a method of reversing *in vitro* the differentiation of differentiated cells of a cell line or obtained from the tissue of an animal or a plant to produce a desired type or types of totipotent or pluripotent stem cell(s) or stem cell line(s), which comprises providing isolated RNA comprising RNA extractable from the desired type(s) of stem cell or stem cell line to a cell culture of said differentiated cells whereby the desired reversal of differentiation of the differentiated cells into said type(s) of stem cell or stem cell line type(s) is achieved.

As existing methods for isolating stem cells are often laborious and require large amounts of material from a subject, the ability to reverse the differentiation of differentiated cells to provide stem cells provides a more convenient alternative which is less time consuming, more economical and less invasive. In particular, where it is desired to obtain stem cells from a subject suffering from a disorder it simply may not be practical to isolate stem cells directly from such a subject due to the invasive nature of the procedure needed to recover stem cells or the limited amount of material recoverable from the patient. The method of the invention also has the advantage that a wide range of stem cells can be obtained and that the stem cells obtained have the capacity to differentiate into a wide range of differentiated cell types.

The differentiated cells employed in the method may be any suitable differentiated cells including any mentioned herein. In particular, the differentiated cells may be cells that are readily accessible. The differentiated cells may be obtained from skin samples or from the bucal cavity. In a particularly preferred case the differentiated cells may be fibroblasts and particularly skin fibroblasts. In some cases the cells may be obtained from a bodily fluid and in particular from blood. In some cases white blood cells may be used such as, for example, lymphocyres.

The RNA may be provided using any of the methods described herein. After provision of the RNA to the cells the resulting stem cells may be cultured and passaged. The reversal of differentiation may be confirmed by examining cell morphology and by checking for the presence of stem cell specific markers. The ability of the stem cells for self-renewal may also be confirmed with the cells being passed through several passages to check that no differentiation occurs. The ability of the cells to differentiate into specific cells may also be examined. The karyotype of the obtained stem cells may be determined and in particular it may be checked to ensure that the karyotype of the cells is stable over several generations. The stem cells may be expanded. Samples of the stem cells may be frozen for later use or reference. In particular, samples of cells that have undergone low numbers of passages may be frozen, such as cells that have undergone ten or less, five or less, two or one passage(s). Clonal stem cell lines may be established from the general

stem cell population and selected for specific desired characteristics such as their developmental capacity.

The resultant stem cells may also be manipulated to introduce desired genetic modifications. For example, if the original differentiated cells comprised a genetic defect the defect may be corrected. Sequences that can functionally compensate for missing or defective sequences may be introduced. Functional copies of missing or defective genes or other sequences may be introduced. Techniques such as PCR and Southern blotting may be used to screen for and identify clones with the desired modifications. The obtained stem cells may be differentiated and then assessed to check that the defect has been corrected. Techniques such as gene targeting may be used to introduce site-specific changes to the endogenous copies of genes. These may be employed in conjunction with site-specific recombinases to remove selectable markers used in the targeting. In particular, single gene disorders may be corrected using such techniques. Both dominant and recessive disorders may be corrected.

The stem cells obtained may be used in any of the aspects of the invention that utilise stem cells. They may also be used in any of the other applications of stem cells. They may, for example, be used in the generation of non-human chimeric animals and hence transgenic non-human animals

The invention provides cells obtained using the above methods. The cells may be provided in some cases as frozen aliquots in suitable receptacles. The invention also provides cell extracts of the cells.

In vitro methods for inducing the differentiation of stem cells

The invention also provides methods for inducing the differentiation of stem cells *in vitro*. The differentiation is achieved by providing the cell with an RNA comprising an RNA extractable from the cell type that it is desired to differentiate the stem cell into. In particular the invention provides a method of inducing *in vitro* totipotent or pluripotent stem cells of a stem cell line or obtained from a tissue of an animal or plant to differentiate into one or more desired cell types, which comprises providing isolated RNA comprising RNA extractable from tissue or cells comprising said desired cell type(s) to a cell culture of said stem cells under conditions whereby the desired differentiation of said stem cells is achieved.

Any stem cell may be used in the methods, including any of those mentioned herein. In a preferred embodiment, the stem cells to be differentiated may be obtained using the methods of the invention to reverse the differentiation of differentiated cells to provide the stem cells. In cases where the differentiated cells obtained are intended for use in the manufacture of medicaments to treat a subject, the stem cells may originate from the intended recipient. In some cases the stem cells may originate from a recipient who has a genetic defect and preferably the genetic defect may have been corrected or ameliorated in the stem cells in such cases.

The RNA may be provided to the target stem cells using any of the methods discussed herein.

The stem cells may be induced into any desired cell type including any of those mentioned herein. In a preferred case the stem cell will be differentiated into a stable terminal differentiated cell type. A terminal differentiated cell type may generally be considered as one that does not naturally differentiate to give any other cell type and is typically at the end of a lineage. In some cases the stem cell may be differentiated into an intermediate cell between the stem cell and the terminal cell of the lineage. Such intermediates may have some degree of proliferative capacity.

The differentiated cell may be one of an organ or tissue such as the liver, spleen, heart, kidney, skin, gastrointestinal tract, eye, or reproductive organ. In a preferred embodiment the differentiated cell type may be one that is missing, present in reduced number or defective in a particular condition. The condition may be any of those mentioned herein and include injury, degenerative disease or a condition resulting from a genetic disorder. In a particularly preferred embodiment the differentiated cell may be an islet of Langergans cell as the resulting cells can be used to treat diabetes. In another case the differentiated cell may be one of the central nervous system that can be used to treat a disorder or injury of the nervous system and particularly a disease of the brain or a spinal cord injury. In a preferred embodiment bone marrow stromal cells may be differentiated into neuronal cells.

In some cases the stem cell that is differentiated may be a puripotent, but not totipotent, stem cell. In such cases the stem cell may, for example, be differentiated into a cell type that the stem cell is known to differentiate into in the organism it is isolated from.

In a preferred embodiment, bone marrow stromal stem cells may be differentiated into neuronal cells. In particular, they may be differentiated into neuronal cells expressing neuronal marker proteins (NeuN). Typically, the bone marrow stem cells may be differentiated into neuronal cells by providing an isolated RNA comprising RNA extractable from one or more types of brain cells or brain cell lines. In some cases the RNA may comprise an RNA extractable from brain tissue and in particular it may comprise an RNA extracted from a brain tissue. In a particularly preferred case the RNA may comprise RNA extractable from cortical neurones or a cortical neurone cell line. In some cases RNA extractable from neurones found in other locations than the brain may be employed or from cell lines derived from such neurones.

In another preferred embodiment, bone marrow stem cells may be induced to differentiate into muscle cells and in particular into skeletal muscle cells. Typically the RNA provided will comprise an RNA extractable from muscle cells or muscle cell lines and in particular from muscle stem cells.

The invention provides cells obtained using the above methods. The cells may be provided in some cases as frozen aliquots in suitable receptacles. The invention also provides cell extracts of the cells.

In some cases the stem cells may be present in or on a structure such as a support, membrane, implant, stent or matrix when they are differentiated or alternatively the differentiated cells may be added to such a structure. The structure may then be used in the manufacture of a medicament for treating any of the conditions mentioned herein. Mixtures of different differentiated cell types may also be made, for example, to mimic populations occurring together *in vivo*

In one preferred embodiment the *in vitro* method may comprise:

- providing a stem cell population and culturing it *in vitro* according to established protocols;
- extracting RNA from a desired target tissue type (for example neurones, glia muscle or any of the differentiated cell types mentioned above);
- providing RNA to the stem cells either 1) as naked RNA extract 2) via liposome mediated transfer 3) by electroporation of recipient cells or other established methods; and

- maintaining the cells in culture.

Preferably the resulting differentiated cells may then be formulated into a medicament which can be administered to a subject by an appropriate route such as via the sub-cutaneous, sub dermal, intra-venous or intra peritoneal routes.

Medicaments and methods for treating subjects

The stem cells, RNA and differentiated cells provided by the invention may be used in the manufacture of medicaments to treat a number of disorders. In particular, the RNA and cells of the invention may be used in the manufacture of medicaments for use in improving or rectifying tissue or cellular damage or genetic disease.

The invention may employ a number of approaches to provide medicaments to treat such disorders. In particular, administration of the medicaments of the invention to a subject to be treated may result in:

(a) administration of an RNA of the invention to the subject in order to induce differentiation of stem cells *in situ;*

(b) administration of stems cells obtained by the invention to a subject; and/or

(c) administration of differentiated cells obtained using the methods of the invention to the subject.

Combinations of the above integers may also be employed. For example, medicaments comprising the stem cells may be administered to the subject and then a medicament comprising an RNA capable of inducing differentiation in accordance with the invention may be administered in order to induce their differentiation *in situ*.

In some cases it may be desired to use the methods of the invention to provide a cell type which is missing, depleted in number or functionally defective. The cells of the invention may be provided to a specific site or to a larger region. For example, the cells may be provided to a site of tissue or organ damage or injury such as a wound or broken bone. The cells may be provided to the site of a nerve injury and in particular to a spinal column injury. The cells may be provided to a damaged or diseased liver, kidney, heart or other organ. In the case of damaged or defective cardiac muscle disease such as in heart disease, dead or damaged cells can be

augmented or replaced. Similarly cells can be provided to subjects with liver disease such as liver fibrosis, or other types of liver damage. Typically differentiated cells obtained using the methods of the invention will be provided, in some cases however stem cells obtained using the methods of the invention may be provided and allowed to differentiate *in situ*.

The invention may be used to treat or ameliorate degenerative brain disease, brain or spinal cord injury or other neuronal disorders. In preferred embodiments the cells may be provided to a subject suffering from a degenerative disease and in particular an age related degenerative disease. The disease or damage to be treated with the medicaments of the invention may affect the brain. The subject may, for example, be suffering from a degenerative brain disease. Examples of brain disorders include, in particular, Parkinson's disease, Parkinsonian type disorders, Alzheimer's, dementia, other age related brain pathologies and Motor neurone disease. Multiple sclerosis may also be treated. Another disorder that may be treated is diabetes and particularly type 1 diabetes, by providing insulin producing islet of langerhans cells to replace or augment the defective cells. The invention may also be used for subjects suffering from disorders caused by damage to joints such as, for example, arthritis.

In some case stem cells obtained using the methods of the invention may be administered to the subject, rather than differentiated cells. The stem cells may be administered to augment those already present in the subject. In some cases the stem cells may be administered to a site of tissue damage and then allowed to differentiate naturally. In some case stem cells may be added to augment those already present as the additional stem cells lack some defect present in the resident stem cell population and in particular a genetic defect. For example, the subject may have a genetic disorder that results in the absence of a particular cell type or cell lineage, a reduction in number of a particular cell type or cell lineage or in a particular cell type or lineage being defective. Stem cells lacking the defect may then be transferred to compensate for the genetic defect as they can give rise to the desired cell type or lineage or so that the cells or lineages they give rise to lack the functional defect. The stem cells administered may proliferate to maintain their number and also give rise to differentiated cells and hence have a long lasting effect reducing the need for

frequent treatment. Indeed the transfer of the stem cells may result in a permanent cure or amelioration of the condition.

A subject may, for example, have an immunodeficiency caused by a genetic defect. Transferring a population of stem cells obtained using the invention that do not have the defect may be enough to treat the disorder as a proportion of the immune cells generated will lack the defect and be functional. In some cases the disorder may result from an in infection and in particular a viral infection and the stem cells may have some modification that prevents the cells becoming infected. In other cases stem cells obtained using the methods of the invention may be transferred to subjects whose own stem cell population has been depleted. For example, the subject may have been exposed to radiation or chemical agents that result in a decrease in stem cell number.

In a preferred embodiment of the invention, in cases where stem cells are transferred to a subject they will be derived from the same subject using the invention to produce stem cells from their differentiated cells. In other cases, the stem cells may be differentiated from an immunologically compatible unrelated individual. In some cases, the differentiated cells used to obtain the stem cells may be from a different individual, but the RNA provided to the cells may be from the intended recipient or a genetically compatible recipient. The provision of the RNA may result in the stem cells being immunologically compatible to the intended recipient.

In some cases the medicaments and methods of the invention may involve the RNAs of the invention being provided to the target stem cells *in situ*. This may result in resident stem cells differentiating to give rise to the desired differentiated cell type. Such an approach may be used for any of the above-mentioned conditions and disorders. In such an approach the RNA will typically be delivered so that it only affects a relatively localised population of stem cells. Preferably, the stem cells targeted may be those that give rise to the particular cell type involved in the disorder, but this may not always be the case. For example, the subject may have an immune system disorder and haematopoietic stem cells may be targeted.

Delivery to the chosen population of stem cells may be achieved by providing the RNA locally, such as to the appropriate tissue or organ. The RNA may, for

example, be provided by local injection. The RNA may be provided by injection into a blood vessel or other vessel that leads to the desired target site. The RNA may be administered by local injection to the desired tissue. The RNA may be administered by any of the routes mentioned herein such as intra-muscular injection or by ballistic delivery. In some cases the localised delivery may be achieved because the RNA is provided in a form that specifically targets the RNA to the chosen cells. For example, the RNA may be provided in liposomes or other particles that have targeting molecules for the specific desired stem cell type. In preferred embodiments the RNA may be administered via direct organ injection, vascular access, or via intra-muscular, intra-peritoneal, or sub-cutaneous routes

In one preferred embodiment administration of an RNA is achieved as follows:

- an RNA extract is prepared from desired tissue type including any of those mentioned herein;
- the RNA is injected either directly to affected organ or via systemic delivery as defined above; and
- the RNA induces resident stem cell differentiation resulting in, for example, proliferation of the desired cell type, migration and repair.

In a further embodiment differentiated cells obtained using the invention may be administered to the subject. In a preferred embodiment, the stem cells used to obtain the differentiated cells may have been obtained or derived from the intended recipient. Any of the differentiated cell types mentioned herein may be administered and the subject may be suffering from any of the disorders and conditions mentioned herein.

The differentiated cells may be administered to the localised site affected by the disorder. For example, they may be delivered to the pancreas in the case of diabetes, to the spinal nerve in the case of spinal injury, to the brain for brain disorders and so on. In some cases the differentiated cells may be provided to the subject present on, or in as part of a structure. For example, stents coated with differentiated cells may be inserted into a blood vessel or liver cells may be provided on a matrix to a damaged or diseased liver.

The invention also provides a method of improving or rectifying tissue or

cellular damage or a genetic disease in a subject, the method comprising inducing totipotent or pluripotent resident stem cells in the subject to differentiate into one or more desired cell types, which method comprises providing isolated RNA comprising RNA extractable from tissue or cells comprising said desired cell type(s) to the resident stem cells *in situ*, whereby the desired differentiation of said stem cells is achieved.

The invention also provides a method of improving or rectifying tissue or cellular damage or a genetic disease in a subject, the method comprising administering to the subject an effective amount of differentiated cells obtained *in vitro* by inducing totipotent or pluripotent stem cells of a stem cell line or obtained from a tissue of an animal to differentiate into one or more desired cell type(s), which comprises providing isolated RNA comprising RNA extractable from tissue or cells comprising said desired cell type(s) to a cell culture of said stem cells under conditions whereby the desired differentiation of said stem cells is achieved. In a particularly preferred method the stem cells used are obtained from the subject to be treated. In an even more preferred embodiment, the stem cells used are obtained by using the methods of the invention to induce the reversal of differentiation of differentiated cells *in vitro* to provide the stem cells and in particular the differentiated cells used to obtain the stem cells are obtained from the subject.

The invention also provides an agent for improving or rectifying tissue or cellular damage or a genetic disease, the agent comprising the RNA or differentiated cells as defined herein.

In the above methods of treating a subject the stem cell, differentiated cells, RNA, method of providing the RNA and other aspects may be as defined anywhere herein. In respect of the above agents, the RNA or differentiated cell may be any defined herein.

Cells and pharmaceutical compositions

The invention provides cells obtained by the methods of the invention. The cells may be provided as frozen cells in a suitable receptacle. The cells may be provided in culture. Extracts of the cells are also provided such as whole cell extracts.

The invention also provides pharmaceutical compositions comprising the various RNA molecules, stem cells, and differentiated cells of the invention. The RNA molecules, stem cells and differentiated cells may be formulated with standard pharmaceutically acceptable carriers and/or excipients as is routine in the pharmaceutical art. Techniques for formulating cells and nucleic acids may be employed as appropriate. The cells or RNA may be provided in physiological saline or water for injections. The exact nature of a formulation will depend upon several factors including the particular substance to be administered and the desired route of administration. Suitable types of formulation are fully described in Remington's Pharmaceutical Sciences, 19th Edition, Mack Publishing Company, Eastern Pennsylvania, USA, the disclosure of which is included herein of its entirety by way of reference.

A therapeutically effective amount of the medicament, compositions, cells or RNA molecules will be administered to a subject. The dose may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. A physician will be able to determine the required route of administration and dosage for any particular patient. The dose may be determined taking into account the age, weight and conditions of the subject to be treated, the type and severity of the degeneration and the frequency and route of administration.

The following Examples illustrate the invention.

<u>Example 1:</u> <u>Production of neural and muscle cells from bone marrow stromal stem cells</u>

Marrow harvest and culture.

Bone marrow stromal (mesenchymal) stem cells were obtained from adult Sprague Dawley rats. The technique is based upon the protocol of Owen and Friedenstein (1988), and represents a typical established adult stem cell source suitable for expansion *in vitro*. Briefly, after schedule one killing (cervical dislocation), tibia and femora were excised within 5 minutes of death. All connective

and muscular tissue was removed from the bones and all further procedures were conducted under sterile conditions.

Marrow was expelled from the bones by flushing the bones with media (α-MEMS – Gibco Invitrogen Co. UK) containing 10% foetal calf serum, and 1% penicillin/streptomycin. Flushing was achieved by inserting a 25-guage needle attached to a 5ml plastic barrel into the neck of the bone (cut at both distal and proximal end) and expelling 2ml of media through the bone. The media and bone marrow sample were collected in sterile universal containers. Bone marrow cells were subsequently dissociated by gentle trituration through a 19-guage needle approximately 10 times. One ml of aspirate was then placed in six well plates (SLS Ltd. UK). Two ml of fresh α-MEMS was then added to each well giving a plating density of approximately 12,000-15,000 cells per ml. Plates were then incubated at 37°C, in 5% CO_2 in air and left undisturbed for 24 to 48 hours (Harrison & Rae, 1997).

Following this time period, marrow derived stem cells were isolated from non-plastic adherent cells by aspirating the culture media from the plate. Plastic adherent marrow stromal stem cells remained, and were supported by the addition of 2ml of fresh α-MEMS (10% foetal calf serum and 1% penicillin/streptomycin). New media was applied every 48 hours until the plate was confluent with colony forming units (CFU's) confirmed by microscope analysis (Owen & Friedenstein, 1988, *supra*). Under optimal conditions this required 5 to 7 days at 37°C. Resultant cells were confirmed as stromal stem cells morphologically and immunohistochemically.

RNA procedure

Brain homogenate was prepared and RNA separated using an RNA commercial separation kit or standard phenol based procedures. In the initial experiment, RNA was prepared by a cold phenol extraction method. Brains were freshly dissected from eight freshly killed rats. Eight grams of brain, excluding the cerebellum, was weighed and 5ml of phosphate buffered saline (PBS) was added. The mixture was homogenised in a glass Teflon homogeniser for approximately 4 minutes. An equal volume of 95% saturated phenol was added. The resultant solution was left at room temperature for 15 minutes then centrifuged at 18,000rpm in an ultra

centrifuge for 30 minutes. The aqueous phase was retained and brought to a concentration of 0.1M $MgCl_2$ by the addition of 1M $MgCl_2$. Two volumes of ethanol were then added and precipitation was allowed to occur for approximately 30 minutes. A final spin at 6,000rpm for 15 minutes produced an RNA rich precipitate, which was retained and stored under ethanol. Resultant RNA was air dried and dissolved in 6ml of fresh media as defined above.

One ml of media containing the RNA was added to each well of confluent bone marrow stem cells for 24 hours. After 24 hours the RNA media was removed and replaced with fresh media. Cells were observed for phenotypic change every 12 hours.

Further, cells were subjected to immunohistochemical analysis to confirm that the RNA induced in the bone marrow stem cells was a neuronal phenotype. This was achieved by testing treated cells for the expression of a neuronal marker NeuN. The results obtained are indicated in the Table below.

	Morphology	**NeuN**
Untreated cells	Retained CFU morphology	-
Brain RNA treated cells	Developed Neuronal type Morphology	+

Examination of the cells showed the RNA induced change in cellular differentiation to a clear neuronal phenotype 24 hours after application of brain derived RNA. Untreated bone marrow stem cells retained the classic colony forming unit morphology. However, as early as 12 hours post-treatment the brain RNA treated stem cells showed typical neuronal and glial morphologies. Further, these cells expressed a commonly used immunochemical marker for neurones. Control cells did not. This change in phenotype survived passage (x3) and thus would appear a stable change in recipient stem cell differentiation. That donor tissue RNA was responsible for the change in stem cell differentiation was confirmed by subsequent experimentation in which the inductive effect of RNA was abolished by pre-

treatment with RNAze, yet remained resistant to treatment of the donor brain RNA with trypsin, a potent protease.

The experiment was repeated using donor RNA, derived from skeletal muscle to confirm the specificity of the induced differentiation. It was clearly visible that the stem cells prepared as above and treated with muscle derived RNA (prepared using a commercially available kit, RNAzol), showed a stable differentiation change to muscle phenotype. This was confirmed by immuno staining with Phospholamban and Phalloidin. In the muscle study, the stem cells were exposed to muscle derived RNA (derived with a different RNA separation technique) via a different method of RNA delivery. RNA was delivered to the stem cells via liposomes prepared after the methodology of Felgner *et al* (1987, P.N.A.S., **84**, 7413-7417). Thus it can be concluded from these studies that the induction in stem cells is specific to the donor tissue source, and that the RNA can be added to the stem cells via a variety of techniques commonly employed to deliver nucleic acids to cells.

Example 2: The effects of brain RNA differentiated stem cells on age related damage to the rat brain, assessed by spatial learning and memory performance of recipient animals.

Bone marrow mesenchymal stem cells were prepared *in vitro* as described above in Example 1. When the cells reached confluence, they were exposed to brain RNA (prepared as above) for 12 hours. Donor stem cells were derived from a pigmented rat strain (Lister Hooded). Donor RNA and recipient animals were provided from a different strain of rats (Sprague Dawley).

Recipient Sprague Dawley rats were ex-breeder male rats aged between 468-506 days. It is well established that such animals of advanced age cannot learn to locate a hidden platform in a water maze (Stewart & Morris, 1993; Bagnall & Ray, 2000). Experimental animals received a 0.5ml intra-venous injection of brain RNA treated stem cells, equating to the product of one six well plate of brain RNA treated cells. Control animals received an equivalent amount of untreated stem cells. Briefly, cells were collected from plates, either treated (experimental) or untreated (control) by mechanically removing them from the plastic plates using a rubber policeman and collected, by aspiration, in culture media. Cells were concentrated via a 5-minute

spin at 1000rpm and brought to a concentration outlined above. All injection procedures were conducted blind. For both groups, injections were mediated via the tail vein.

Fourteen days after injection, the aged rats were assessed blind on a commonly used spatial learning task, the Morris water maze. Each animal received 3 swims per day over a 3 day period with an inter trial interval of 10 minutes (Stewart & Morris, 1993). Latency to find the platform on each trial was recorded for each animal. Each trial consisted of a 60 second swim. If after that interval the animal had not located the platform, it was gently guided to the platform by the experimenter. Upon reaching the platform, the animal was allowed 10 seconds to orient to its location prior to removal to the home cage. Learning is evidenced by a decrease in time to locate the platform over repeated trials.

The results of the study are presented in Figure 1. Control rats (n=9) receiving intra venous stem cells which had not been exposed to RNA, could not learn this task with no decrease in response latency over trials. However, the experimental animals receiving brain RNA treated stem cells showed a remarkable learning ability comparable to that of young rodents ($p<0.0000000001$). Two conclusions may be drawn from this study. First, RNA treated stem cells can significantly ameliorate age related deficits in spatial learning. Control untreated stem cells cannot. Second, it should be noted that donated stem cells were from a different strain of rat and recipient animals were not rendered immunodeficient. Thus, the results suggest that not only did the experimental group cells differentiate to appropriate neural tissue capable of functional improvement, they acquired an immunological status rendering them acceptable to the recipient. It should be noted that donor brain RNA was sourced from sibling animals to the recipients, yet donor cells were sourced from a different strain.

The results not only confirm that RNA differentiated stem cells can repair age related damage by restoring behavioural capabilities, but further that such treated cells acquire the immune characteristics of the donor RNA. This offers a strategy to change the immune profile of stem cell lines or stem cell banks to create differentiated cells with specific compatibility with the recipient.

<u>Example 3: *In vivo* stimulation of resident stem cells via exogenous RNA stimulated differentiation, migration and integration.</u>

Given the powerful stimulatory effects of exogenous RNA on stem cells established in Examples 1 and 2, and the effects of these cells on repairing age related damage in a mammalian model, a further Example is given, establishing the effects of primary tissue derived RNA on host animal resident stem cells. To this end, neonate rats received an intraperitoneal injection of donor GFP expressing crude bone marrow at age 1 day postnatal. Each animal received approximately 800,000 cells in a 0.2ml injection. These foreign cells were readily integrated in host bone marrow and were observed to contribute to this biological environment. At age 90 days, GFP bone marrow grafted animals were randomly assigned to two groups.

Experimental animals received an injection of brain RNA, control animals received an injection of physiological saline. Experimental brain RNA was prepared as outlined in Example 1. Injection was conducted sub-cutaneously. Each animal received one whole brain equivalent of donor RNA in a 0.5ml injection. Controls received an equivalent injection of physiological saline.

The results obtained showed a significant thickening of recipient cortex ($p<0.0001$) in experimental animals compared to control animals. Further, a significant number of differentiated neurones and glia in experimental animals showed expression of GFP indicating infiltration of resident bone marrow stem cells into the brain following application of exogenous brain RNA.

<u>Example 4: Induced differentiation of stem cells via exogenous RNA isolated from a primary cell culture of cortical neurones.</u>

A purified culture of embryonic cortical neurones was established in the laboratory following the protocol of Saneto and deVellis (1987). Briefly, time mated Sprague Dawley female rats were sacrificed at day 16 of gestation. The abdominal area was sterilised with 70% alcohol and the uteri exposed. Uteri containing the embryos were then dissected free from the uteri and placed in a large 100mm Petri dish. All the above procedures were conducted on a clean bench outside the sterile hood to prevent contamination. All further procedures were conducted under sterile conditions.

Intact uteri were then washed with physiological saline and transferred to another sterile Petri dish. Embryos were then dissected free from the uteri and placed in a new Petri dish for brain dissection. Brain tissue was exposed and gently removed with a spatula and cortices were dissected under a dissecting microscope. Meninges were then dissected clear in physiological saline. After cortices were processed, they were gently disrupted with repeated passage through a 10ml glass pipette. The cell suspension was then passed through a Nitex 130 filter (mesh size 130μm) and the filtrate centrifuged at 40g. The pellet was then re-dispersed in serum free basal media (Saneto & deVellis, 1987, *supra*) and passed through Nitex 33 (mesh size 33μm) and cells counted.

The suspension was supplemented with insulin (5μg/ml) and transferrin (100μg/ml) to form neurone-defined medium. Cells were seeded at a density of 1 x 10^5 per well on 24 well culture plates pre-coated with polylysine (2.5μg/ml). Cultures are reported as containing more than 95% neurones by immunological criteria of expressing the marker neurofilament protein, while not expressing the biochemical and immunological markers for astrocytes and oligodendrocytes (Saneto & deVellis, 1987, *supra*). Media was changed every third day post plating and cultures were maintained for 12 days prior to RNA extraction.

RNA was extracted from the primary cortical neurone cultures via a commercial kit (RNAzol) using the manufacturer's protocol. Resultant RNA was collected and redissolved in bone marrow culture medium (as defined in example 1) just prior to application to a confluent colony of rat bone marrow cells prepared as in Example 1. Each recipient bone marrow culture well received the total RNA extracted from one complete 24 well primary neuronal culture (although similar results were obtained a wide variety of exogenous RNA concentrations). Bone marrow stem cells were examined microscopically 24 hours after application of exogenous RNA dissolved in media. Control bone marrow stem cells received an equal amount of RNAzol prepared bone marrow stem cell RNA.

Results showed all experimental stem cell wells produced clearly differentiated neurones, which stained positively for neuronal markers. No observable change in stem cell differentiation was found in the Bone marrow RNA

treated wells. These results suggest that donor RNA from a purified cell source may induce highly specific stem cell differentiation.

The differentiation inducing effect of exogenous RNA fractions was sensitive to pre-treating the donor RNA with Rnaze yet insensitive to trypsin. This suggests that RNA mediated the effect. These effects may be repeated using a wide range of RNA doses delivered exogenously by a variety of delivery methods and vehicles including liposomes or electroporation.

Example 5: Retro-transformation of terminally differentiated cells via exogenous application of RNA fractions obtained from stem cell sources

Given the powerful and specific effects of RNA tissue extracts on stem cell differentiation in Examples 1 to 4, a final example of the technology is provided. Here, the donated RNA rich extract is obtained from cultured stem cells. Its ability to reverse differentiation is tested by exogenous application to terminally differentiated adult fibroblasts to investigate if recipient mature differentiated cells could be re-differentiated to stem cell character and behaviour via stem cell derived RNA fractions. The results obtained show that stem cell type tissue may be generated from differentiated tissue.

Adult rat (Lister Hooded) fibroblasts were obtained and maintained in culture conditions according to the protocol of Kawaja *et al.*, (1992). A biopsy of skin (approx. 1 cm^2) was placed into a sterile Petri dish containing phosphate buffered saline (PBS), pH7.4. The biopsy was then dipped (x3) in another dish filled with 70% ethanol then placed back in fresh PBS and cut into 1-2 mm pieces. These explants were placed into 60-mm tissue culture dishes pre-filled with 1ml Delbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS) and 0.1% glutamine. 10 units/ml of penicillin and 100 μg/ml streptomycin were also added. This culture was incubated with 5% CO_2 at 37°C.

After two days in such culture conditions, fibroblasts begin to migrate from the explant, at this stage an additional 2-3 ml of nutrient media was added. When the plates reached approximately 90% confluence, they were passaged by incubating the cultures with 1-2 ml of trypsin solution and transferred to a 15-ml centrifuge tube, then centrifuged in a bench centrifuge for 10 minutes at room

temperature. The supernatant was discarded and the pellet resuspended in 10 ml of culture medium. These cells were maintained in untreated 6 well plates seeded with 0.5ml cell suspension in 2 ml of medium until confluence. At this time they could be further passaged.

Donor RNA was sourced from adult rat bone marrow mesenchymal stem cells maintained in culture as reported in Example 1 or from neural stem cells (neurospheres) cultured according to the protocol of Reynolds & Weiss (1992). All RNA rich extracts were prepared by RNAzol separation following the manufacturer protocol. Thus, two donor RNA fractions were obtained: 1) bone marrow stem cell RNA (BMS-RNA) and 2) neural stem cell RNA (NS-RNA). These fractions were dissolved respectively in fibroblast growth media at various concentrations from 0.75μg/ml to 500μg/ml and added to adult differentiated fibroblasts maintained in final culture wells for 5 days. Transformation of fibroblasts via stem cell derived exogenous RNA appeared across a wide range of doses.

In the results obtained, differentiated fibroblasts with no treatment of exogenous stem cell RNA showed no change in phenotype. 48 hours after RNA application, fibroblasts treated with an exogenous RNA dose of 25μg/ml of either NS-RNA or BMS-RNA both showed a clear change in morphology. Recipient fibroblasts of NS-RNA formed floating spheres with the appearance and characteristics of neurospheres, from these neural phenotype cells began to radiate these could be easily identified as both neuronal and glial in morphology. Recipient fibroblasts of BMS-RNA, at for example 25μg/ml, showed the classical bipolar shape of mesenchymal stem cells and were plastic adherent.

Subsequent experimentation showed these cells to be able to produce neurones and muscle tissues when further induced by exogenous RNA as described in Example 1. The retro-differentiation inducing effect of exogenous stem cell derived RNA fractions was sensitive to pre-treating the donor RNA with Rnaze yet insensitive to trypsin. This suggests that the effect was mediated by RNA. These effects may be repeated using a wide range of RNA doses delivered exogenously by a variety of delivery methods and vehicles including liposomes or electroporation.

Thus, differentiated adult tissue can be retro-differentiated into stem cell like tissues when subjected to various stem cell-derived RNA fractions. The properties of

the resulting cells reflect the donor stem cell morphology, behaviour and potential. Thus a novel and ethically less contentious way of obtaining both totipotent and pluripotent stem cells for a variety of applications in regenerative medicine is provided.

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CLAIMS

1. A method of inducing *in vitro* totipotent or pluripotent stem cells of a stem cell line or derived from a tissue of an animal or plant to differentiate into one or more desired cell types, which comprises providing isolated RNA comprising RNA extractable from tissue or cells comprising said desired cell type(s) to a cell culture of said stem cells under conditions whereby the desired differentiation of said stem cells is achieved.

2. A method as claimed in claim 1 wherein said stem cells are adult animal stem cells or an adult stem cell line.

3. A method as claimed in claim 1 wherein said stem cells are embryonic stem cells or a stem cell line derived from such cells.

4. A method as claimed in claim 2 wherein said adult stem cells are bone marrow stromal cells, haematopoietic stem cells or neuronal stem cells or a corresponding derived stem cell line.

5. A method as claimed in any one of the preceding claims wherein said stem cells are human stem cells or a human stem cell line.

6. A method as claimed in any one of the preceding claims wherein said stem cells are caused to differentiate into one or more stable terminal cell types.

7. A method as claimed in any one of the preceding claims wherein the stem cells are genetically modified prior to differentiation.

8. A method as claimed in any one of the preceding claims wherein the stem cells are derived from the intended recipient of said desired cells.

9. A method as claimed in any one of the preceding claims wherein said RNA comprises RNA extracted from tissue or cells of an individual different from the source of the stem cells, said extracted RNA being derived from a donor having an immunological profile compatible with the intended recipient of the desired cells.

10. A method according to any one of the preceding claims wherein an RNA extract is provided for uptake by the stem cells which is a whole tissue or whole cell RNA extract.

11. A method as claimed in any one of the preceding claims wherein RNA-extractable from one or more types of brain cell or brain cell line is provided for uptake by stem cells.

12. A method as claimed in any one of claims 1 to 10 wherein the stem cells are bone marrow stromal stem cells and the isolated RNA provided comprises RNA extractable from one or more types of brain cell or skeletal muscle or a corresponding derived cell line of either.

13. Use of an RNA capable of inducing differentiation of stem cells in accordance with any one of claims 1 to 12 in the manufacture of a medicament for use in improving or rectifying tissue or cellular damage or a genetic disease.

14. A use according to claim 13 wherein said RNA is suitable for inducing *in vivo* differentiation of totipotent or pluripotent stem cells for treatment of a degenerative brain disease or brain or spinal cord injury.

15. A use according to claim 13 wherein said RNA is suitable for inducing *in vivo* differentiation of totipotent or pluripotent stem cells for treatment of a disease selected from liver disease, heart disease, skeletal or cardiac muscle disease or type I diabetes.

16. A use according to claim 13 wherein differentiation of stem cells is induced *in vivo* to counteract age-related degenerative disease.

17. A method of reversing *in vitro* the differentiation of differentiated cells of a cell line or obtained from the tissue of an animal or a plant to produce a desired type or types of totipotent or pluripotent stem cell(s) or stem cell line(s), which comprises providing isolated RNA comprising RNA extractable from the desired type(s) of stem cell or stem cell line to a cell culture of said differentiated cells whereby the desired reversal of differentiation of the differentiated cells into said type(s) of stem cell or stem cell line type(s) is achieved.

18. A method according to claim 17, wherein the stem cell is as defined in any one of claims 2 to 5.

19. A method according to claim 17 or 18, wherein the differentiated cells are selected from skin cells, bone marrow cells and haematopoietic cells or a cell line derived from such cells.

20. A method according to claim 17 or 18, wherein the differentiated cells are fibroblasts or a fibroblast cell line and the RNA is extractable from bone marrow stem cells or neuronal stem cells.

21. A method according to claim 20, wherein the isolated RNA provided comprises RNA extractable from bone marrow stromal stem cells, neuronal stem cells or a stem cell line derived from either.

22. An *in vitro* method of producing differentiated cells, which comprises:

(c) performing a method according to any one of claims 17 to 21 to produce stem cells or a stem cell line from differentiated cells;

(d) performing a method according to any one of claims 1 to 12 on the stem cells or stem cell line to produce differentiated cells.

23. A method according to claim 22, further comprising introducing a genetic modification into the stem cells.

24. Cells obtained by a method according to any one of claims 1 to 12, or 17 to 23.

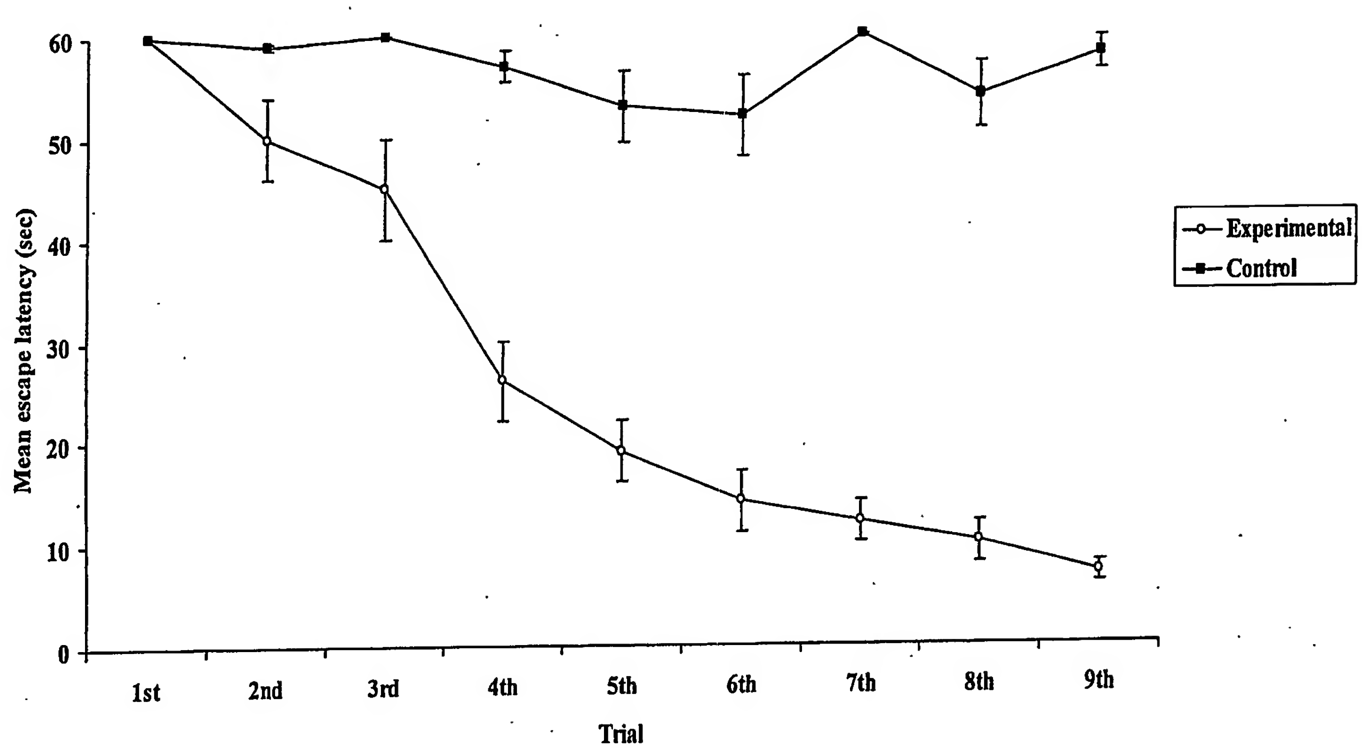
25. Use of cells according to claim 24 in the manufacture of a medicament for use in improving or rectifying tissue or cellular damage or a genetic disease.

ABSTRACT

DIFFERENTIATION METHOD

The present invention provides methods for the control of differentiation. In particular, the invention provides a method for inducing totipotent or pluripotent stem cells of a stem cell line or obtained from a tissue of an animal or plant to differentiate into one or more desired cell types. In addition, the invention provides a method for reversing the differentiation of differentiated cells of a cell line or obtained from the tissue of an animal or a plant to produce a desired type or types of totipotent or pluripotent stem cell(s) or stem cell line(s). Both methods involve the provision of RNA extractable from the desired cell type(s) to the target cells to achieve the require effect. The methods can be perform *in vitro* or *in vivo* and the cells produced have various therapeutic utilities.

Figure 1


Mean escape latency (sec)
60
50
40
30
20
10
0
1st
2nd
3rd
4th
5th
6th
7th
8th
9th
Trial
Experimental
Control

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